

The effects of intraarticularly injected sodium hyaluronate on levels of intact aggrecan and nitric oxide in the joint fluid of patients with knee osteoarthritis

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Summary

Objective: Intraarticular injections of sodium hyaluronate (Na-HA) appear effective in reducing subjective symptoms of osteoarthritis (OA) and may also have protective effects on the cartilage matrix. The present study analyzed the suppressive effects of Na-HA on the release and degradation of aggrecan and on levels of nitric oxide (NO) in the joint fluid of patients with knee OA.

Design: Sixteen OA patients with knee joint effusion were treated by 5 weekly intraarticular injections of Na-HA. Prior to each Na-HA injection, joint fluid was collected to determine the levels of chondroitin 4-sulfate (C4S) and chondroitin 6-sulfate (C6S), intact aggrecan and NO.

Results: One week after the final injection, the joint fluid levels of C4S, C6S, and NO were significantly decreased. In contrast, the joint fluid level of intact aggrecan was stable during the series of Na-HA injections. A trend was seen for a positive correlation ($P < 0.1$) between the clinical score and C4S or C6S joint fluid levels, and for a negative correlation between the joint fluid levels of intact aggrecan and C4S or C6S. No significant correlations were observed between joint fluid levels of NO, the clinical score, and levels of C4S, C6S, and intact aggrecan.

Conclusion: The results of this study suggest that intraarticularly injected Na-HA is able to improve the clinical symptoms of OA partially based on its ability to reduce the release and degradation of aggrecan and/or to enhance the synthesis of aggrecan in the joint tissues of the patients with knee OA. While Na-HA also reduces the NO level in the joint fluid of patients with knee OA, this effect may be independent from the other effects of Na-HA.

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Key words: Aggrecan, Sodium hyaluronate, Knee osteoarthritis, Nitric oxide.

Introduction

Osteoarthritis (OA) is characterized by the progressive erosion of articular cartilage and represents one of the most prevalent diseases in older individuals. The extensive extracellular matrix of hyaline cartilage contains a dense network of composite collagen fibrils composed mainly of type II, together with type IX and XI collagens¹. Contained within this network are large proteoglycans; one of them, aggrecan, has the ability to aggregate with hyaluronan (HA)¹. In adult human cartilage, aggrecan contains a relatively large proportion of the glycosaminoglycans, chondroitin sulfate (CS) and keratan sulfate (KS)². It is well known that the release and degradation of aggrecan is accelerated with the progress of cartilage destruction in the joints of patients with OA¹. Therefore, the amount of aggrecan and/or degree of degradation of aggrecan released into the joint fluid reflects, in part, the pathology of OA^{3–6}.

It has also been suggested that the increased production of catabolic biomolecular mediators, such as nitric oxide (NO), contributes to the pathogenesis of articular cartilage degradation during the development of OA⁷. Because NO suppresses proteoglycan⁸ and collagen synthesis⁹, it is thought to mediate some of the degradative effects of IL-1 on cartilage. NO may be involved in the production and activation of proteases and may modulate the production of metalloproteinases^{10,11}. This suggests that NO might accelerate the proteolytic cleavage of aggrecan. In addition, chondrocytes are known to produce large amounts of NO when they are stimulated by proinflammatory cytokines¹², and the meniscus and synovium are also reported to produce NO^{13,14}.

The intraarticular injection of sodium hyaluronate (Na-HA) has been extensively used in the treatment of OA in many countries. Na-HA appears to be clinically effective as indicated by a reduction in joint pain and improved joint mobility^{15–17}. Various studies on joint tissues¹⁸ and experimental models of arthritis^{19,20} have examined effects of Na-HA on cell function and tissue homeostasis. Na-HA has a protective role against cartilage matrix degradation in experimental OA²¹ and in cartilage explants treated with IL-1 or oxygen-derived free radicals^{22,23}. It has been reported that Na-HA stimulates the production of the tissue inhibitor of metalloproteinase-1 (TIMP-1) from bovine articular

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chondrocytes and reduces the stromelysin/TIMP-1 ratio²⁴. Na-HA has also been shown to inhibit two major chondrolytic activities of the fibronectin fragment, the suppression of synthesis of proteoglycan and the enhanced release of stromelysin-1, both *in vitro*²⁵ and *in vivo*²⁶, although in human cartilage Na-HA only delays MMP-3 induction by a week and then the release of MMP-3 increases to non Na-HA treated levels²⁷. This *in vitro* study using human cartilage²⁷ also suggested that Na-HA enhances aggrecan synthesis and reported that this effect was supported by other researchers^{18,28,29}. In addition, it has been demonstrated that Na-HA has a suppressive effect on NO production in the meniscus and synovium during development of OA in animal models^{30,31}.

Based on the results described above, it is speculated that Na-HA may inhibit NO production from the synovium and menisci and may also down-regulate the activity of proteolytic factors, thus resulting in an increased level of intact aggrecan in the knee joint cartilage of patients with OA. In this study, the inhibitory effects of Na-HA on the release and degradation of aggrecan were examined by measuring the CS levels and the level of intact aggrecan in joint fluid of patients with knee OA. In addition, the effect of Na-HA on the level of NO was also investigated. The relationship among the parameters studied was further analyzed to clarify the mechanism of Na-HA activity.

Materials and methods

PATIENT INCLUSION/EXCLUSION CRITERIA

Patients with OA were selected according to previously described criteria¹⁷. Inclusion criteria were: idiopathic OA of the knee as defined by validated clinical and X-ray criteria showing a sensitivity of 91% and a specificity of 86%³²; an age range of 50–70 years; and the informed consent of the patient. All patient-related procedures were approved by the Institutional Review Board of Inoue Memorial Hospital. Exclusion criteria were: inflammatory joint diseases, ESR > 40 mm after 1 h or RF > 1:40, respectively; specific arthropathies, for example, chondrocalcinosis; excessive joint effusion (> 40 ml); excessive obesity (Broca's formula +50%); severe axis deviations or instabilities; joint prosthesis of the lower limbs; symptomatic OA of the hip ipsilateral to the index knee; intraarticular injection, for example, the use of corticosteroids within 3 months prior to testing; infectious or febrile diseases; joint or skin infections; and pregnancy. Patients were also required to abstain from other disallowed medications, for example, other intraarticular injections, corticosteroids, 'chondroprotective' agents, NSAIDs, and analgesics other than occlusive dressings. Other established treatments were allowed to continue unaltered during the whole study period and were recorded.

PATIENT PROTOCOL/SAMPLE COLLECTION

Between April 2001 and February 2002, 16 patients (four males and 12 females, mean age: 59.1 years) with knee OA with joint effusion underwent 5 weekly intraarticular injections of Na-HA in the Department of Orthopaedics at the Inoue Memorial Hospital. Diagnoses of OA were made according to the criteria described above. These patients were treated by 5 weekly intraarticular injections of 1% Na-HA (800 kDa) solution, kindly provided by the Seikagaku Corporation (Tokyo, Japan), with a dosage of 2.5 ml/injection. On admission and before each Na-HA injection, as much joint fluid as possible was collected by using

a 19-gauge needle attached to a disposable injection syringe. The joint fluid specimens were transferred into plastic tubes, centrifuged at 10 000g for 15 min to remove cells and tissue debris and stored at –80°C until analyzed.

EFFICACY OF NA-HA: CLINICAL SYMPTOMS

The clinical efficacy (clinical score) of Na-HA injected into a knee joint with OA was studied by summing the scores for joint pain and inflammatory parameters at the start of the study (baseline) and at every weekly visit (assessment score)³³ for each patient. The clinical assessment of the OA knee joint was always obtained prior to joint fluid aspiration and Na-HA injection. Briefly, the degree of joint pain was based on three different categories, pain at rest, pain in motion and pain in passive motion, using a four-step rating scale of 0 = none, 1 = mild, 2 = moderate, and 3 = severe. The degree of joint inflammation was based on three different categories, swelling, floating patella and local warmth, using a four-step rating scale of 0 = none, 1 = mild, 2 = moderate, and 3 = severe. The improvement in both degree of joint pain and joint inflammation was evaluated by comparing the clinical scores at baseline with the assessment score at weekly intervals for all patients.

The examination was performed blindly by two observers and averaged to minimize observer bias. Because this is an open label study, caution must be used in interpreting any clinical data. The biochemical analyses described below were performed separately from the clinical assessments. The researchers analyzing each joint fluid sample were blinded to the results of the clinical assessment.

QUANTIFICATION OF CHONDROITIN 4- AND CHONDROITIN 6-SULFATE

Chondroitin 4-sulfate (C4S) and chondroitin 6-sulfate (C6S) levels in the joint fluids were analyzed as previously described³⁴. The joint fluids were first treated by a series of digestions with chondroitinase ABC and AC-II (Seikagaku Corporation). The chondroitinase digestions produce the unsaturated disaccharides, Δ Di-4S and Δ Di-6S from the structure of C4S and C6S in CS chain. After ultrafiltration of the digested solutions, the levels of Δ Di-4S and Δ Di-6S in the filtrates were analyzed by high performance liquid chromatography (HPLC).

DETERMINATION OF THE LEVEL OF INTACT AGGREGAN

For determining the amount of aggrecan in joint fluid we used an enzyme-linked immunosorbent assay (ELISA) system based on the ability of intact aggrecan to bind to HA. Micro-titer plates (96-well, Nalge Nunc International, Tokyo, Japan) were coated with anti-KS antibody (5-D-4, Seikagaku Corporation) dissolved in phosphate buffered saline (PBS) at pH 7.4 at a concentration of 20 μ g/ml and incubated overnight at 4°C. The plates were washed twice with PBS and then blocked (Immunoassay Stabilizer, Advanced Biotechnologies Inc., Epsom, UK) for 2 h at room temperature. The plates were then washed three times with PBS containing 0.05% Tween-20 (Wako Pure Chemical Industries, Osaka, Japan) (washing solution); this washing procedure was used after each of the following steps. The joint fluid was diluted 10 or more times with PBS containing 1% bovine serum albumin (BSA, Seikagaku Corporation) (dilution buffer). The series of standard solutions of intact aggrecan was prepared by dissolving and diluting the intact

aggrecan, which was isolated from bovine nasal cartilage (kindly provided by Seikagaku Corporation), by the dilution buffer. The standard solutions and the diluted joint fluids were separately added to wells of the 5-D-4 coated micro-titer plates and then incubated for 1 h at 37°C. Because unbound 5-D-4 can bind with KS in the hyaluronan-binding protein (HABP), in order to mask any excess, unbound 5-D-4, a 1 µg/ml solution of keratan poly-sulfate (kindly provided by Seikagaku Corporation) dissolved in the dilution buffer was added to each well and the plates were incubated for 1 h at 37°C. A solution of biotin-conjugated HABP (Seikagaku Corporation) was added into each well at 0.3 µg/ml and the plates were incubated for 1 h at 37°C. Then, a solution of avidin-D conjugated with peroxidase (horseradish peroxidase avidin-D, Funakoshi, Tokyo Japan), diluted 10 000 times with the dilution buffer, was added to each well and the plates were incubated for 30 min at 37°C. A substrate solution (TMB peroxidase substrate ELISA, Moss Inc., Pasadena, MD, USA) was then added to each well and the plates were incubated for 15 min at 37°C. Finally, a solution of 1 N HCl was added in order to stop the enzyme reaction, and the absorbance of each well at 450 nm was measured by a plate reader (Well reader SK601, Seikagaku Corporation). The concentration of intact aggrecan in each sample that was able to bind to the HABP was determined using the standard curve generated by the diluted standards. Each specimen was analyzed in duplicate, and the obtained mean value was reported as the result of an individual specimen.

The aggrecan, having its G1 domain (HA binding domain), G2 domain and KS-rich region, can be detected by the method described above. This aggrecan, namely aggrecan aggregating with HA, was defined as "intact aggrecan" in this study.

QUANTIFICATION OF NITRITES

The NO level of the collected joint fluids was estimated by determining the total concentration of NO_2^- and NO_3^- . The joint fluid was diluted with distilled water and then analyzed by a flow-injection system for NO_2^- and NO_3^- determination (Tokyo Kasei Kogyo, Tokyo Japan). Briefly, the diluted joint fluid sample was first passed through a Cd–Cu reducing column (Tokyo Kasei Kogyo). Under these conditions, NO_2^- in the sample is eluted as it is whereas NO_3^- is reduced to NO_2^- . The effluent from the column was then mixed with Griess reagent solution and then passed through a reaction coil with a length of 10 m in a reaction oven setting at 40°C. The Griess reagent reacts with NO_2^- and forms chromogens. The total concentration of NO_2^- and NO_3^- was determined by a UV/VIS detector setting at 570 nm.

STATISTICAL ANALYSES

The results are expressed as the mean \pm S.E.M. of specimens obtained from 14 to 16 patients. The statistical analyses were performed using the paired two-tailed *t* test comparing the values at baseline with each time point. A correlation analysis was also performed among the clinical scores and the joint fluid levels of C4S, C6S, intact aggrecan and NO.

Results

CLINICAL SCORE

The improvement in both degree of joint pain and joint inflammation was evaluated by comparing the clinical

scores at baseline with the assessment score at weekly intervals for all patients. Following 5 weekly intraarticular injections of Na-HA into a knee joint with OA, a significant improvement in the clinical score for the degree of joint pain and joint inflammation (baseline visit: 10.8 ± 0.5 ; 5-week visit: 6.8 ± 0.8 , $P < 0.001$) was observed (Fig. 1). A significant reduction in the clinical score was recorded at every evaluation point when compared to the baseline, pre-treatment clinical score.

CS LEVELS

In this study, the measurement of the alteration of each biochemical parameter was based on its concentration in the original joint fluid. The average volume of joint fluid collected decreased from 13 ml at baseline to 10 ml at the 5-week time point, but this difference was not statistically significant.

The levels of CS in the joint fluids are represented by the concentrations of the unsaturated disaccharides, $\Delta\text{Di-4S}$ and $\Delta\text{Di-6S}$, found after chondroitinase digestion. The concentrations of these disaccharides were both decreased by the series of Na-HA injections, with significant differences observed at the 5-week time point (1 week after the final injection) ($\Delta\text{Di-4S}$: 18 ± 1 nmol/ml at baseline; 15 ± 1 nmol/ml at 5 weeks, $P < 0.05$) [Fig. 2(A)] and ($\Delta\text{Di-6S}$: 56 ± 6 nmol/ml at baseline to 41 ± 4 nmol/ml, at 5 weeks, $P < 0.05$) [Fig. 2(B)].

INTACT AGGREGAN LEVELS

The joint fluid level of intact aggrecan (aggregating with HA) was stable during the series of Na-HA injections. The

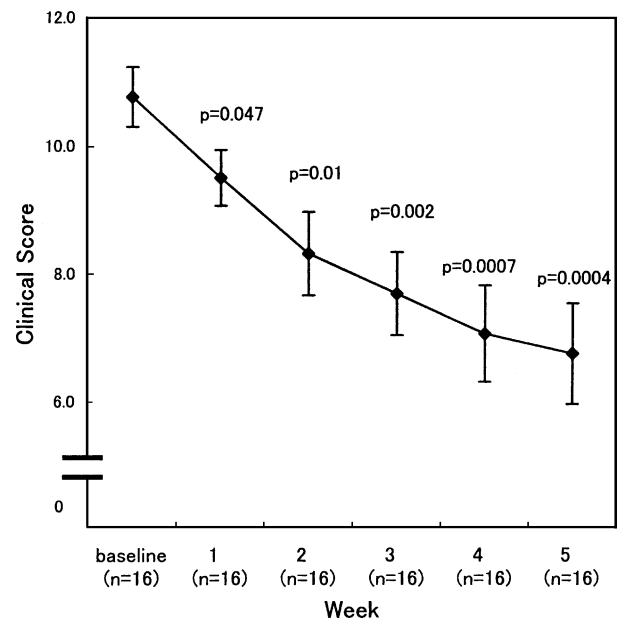


Fig. 1. Efficacy of Na-HA: clinical symptoms. The clinical efficacy (clinical score) of Na-HA injected into a knee joint with OA was studied by summing the scores for pain and inflammatory parameters at the start of the study (baseline) and at every weekly visit (assessment score) for each patient. The clinical assessment of the OA knee joint was always obtained prior to joint fluid aspiration and Na-HA injection. The results are presented as the mean \pm S.E.M. of 16 patients. The *P* values were assessed by comparing the scores from each weekly visit with the baseline values. S.E.M.=standard error of the mean.

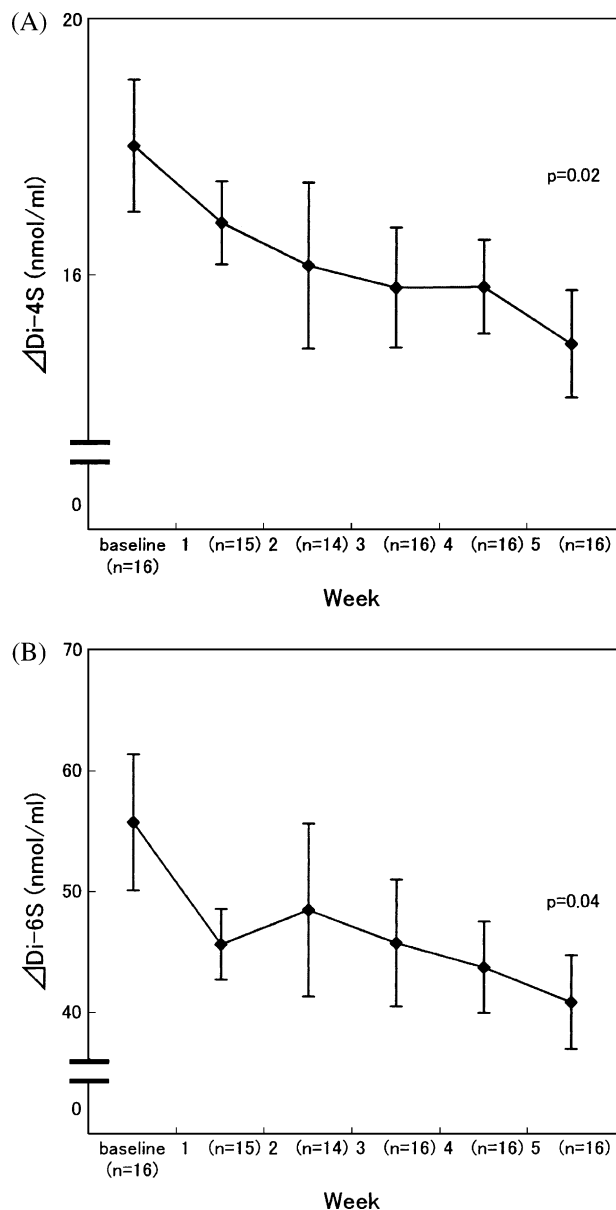


Fig. 2. Joint fluid levels of chondroitin 4-sulfate (C4S) and chondroitin 6-sulfate (C6S). The levels of CS in the joint fluids are expressed as the concentrations of the unsaturated disaccharides, C4S (Δ Di-4S) and C6S (Δ Di-6S) as determined by HPLC analysis after chondroitinase digestion. (A) Δ Di-4S in the joint fluid. (B) Δ Di-6S in the joint fluid; results expressed as the means \pm S.E.M. of 14–16 patients. The P values were assessed by comparing the levels at each weekly visit with the baseline levels. S.E.M. = standard error of the mean.

average value of intact aggrecan was slightly increased by the series of Na-HA injections until the 4-week time point, after which it decreased slightly at the 5-week time point. However, no statistically significant differences were observed at any time point (Fig. 3).

NO LEVELS

Although the deviation at each time point was large, the average joint fluid level of NO gradually decreased after the

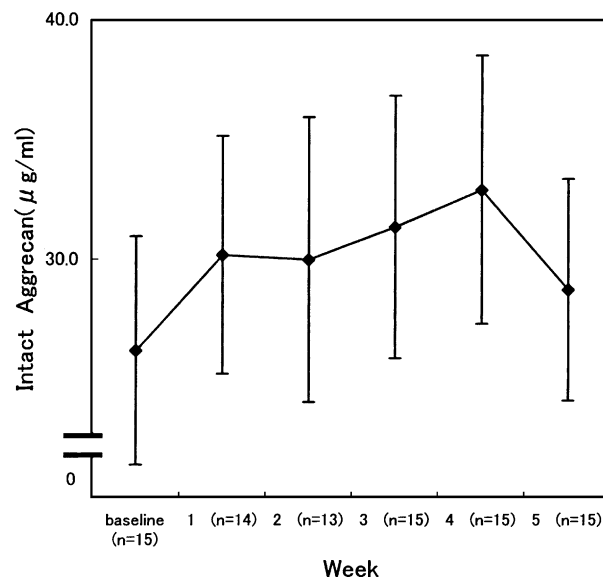


Fig. 3. Joint fluid levels of intact aggrecan. The joint fluid levels of aggrecan that were able to bind to hyaluronan were measured by an ELISA method. The results are presented as the means \pm S.E.M. of 13–15 patients. The P values were assessed by comparing the levels at each weekly visit with the baseline levels. S.E.M. = standard error of the mean.

series of Na-HA injections and was significantly decreased 1 week after the final injection when compared with the baseline level (NO: $100 \pm 16 \mu$ M at baseline; $86 \pm 12 \mu$ M at 5 weeks, $P < 0.05$) (Fig. 4).

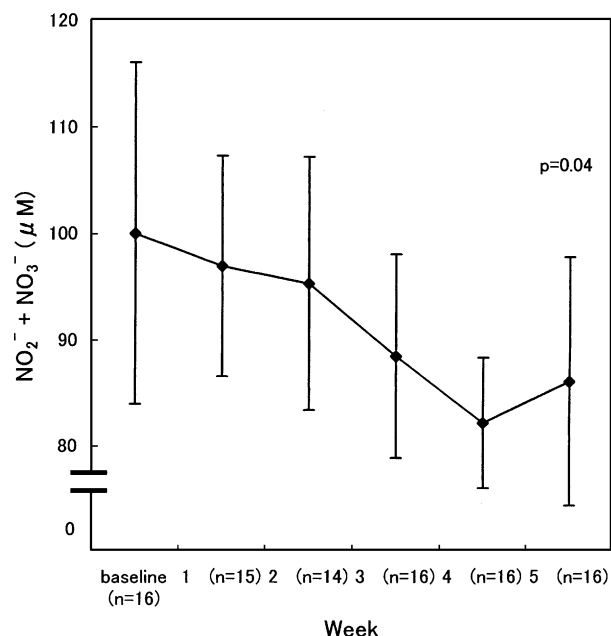


Fig. 4. Joint fluid levels of NO. The NO levels of the collected joint fluids were estimated by determining the total concentration of NO_2^- and NO_3^- . Concentrations of NO_2^- and NO_3^- were determined by the flow-injection analytical method described in the Materials and methods. The results are presented as the means \pm S.E.M. of 14–16 patients. The P values were assessed by comparing the levels at each weekly visit with the baseline level. S.E.M. = standard error of the mean.

CORRELATION ANALYSES

An analysis was performed to identify any correlations among the study parameters: clinical score and joint fluid levels of C4S, C6S, intact aggrecan and NO (Table I). Not surprisingly, a significant correlation ($P < 0.001$) was observed between the joint fluid levels of C4S and C6S. In addition, a trend was identified for a positive correlation ($P < 0.1$) between the clinical score and both C4S and C6S joint fluid levels and for a negative correlation between the joint fluid levels of aggrecan and both C4S and C6S. No significant correlations between joint fluid NO levels and the other study parameters were identified.

Discussion

There are many reports that the intraarticular injection of Na-HA is useful for the treatment of patients with knee OA^{15–17}. Consistent with these reports, we also found significant improvements in three clinical categories of both joint pain and joint inflammation after the series of Na-HA injections (Fig. 1), although caution must be exercised in the interpretation of the clinical findings from this open label study. Further, in this study, we attempted to clarify the mechanism of Na-HA activity, using the joint fluid obtained immediately prior to the Na-HA injections for biochemical analyses.

As shown in Fig. 2, the CS level in joint fluid decreased throughout the series of Na-HA injections, reaching significant levels of difference for both C4S and C6S 1 week after the final injection. Because CS, especially C6S, is a main and relatively specific component of aggrecan in cartilage, this result reflects the reduction of the total aggrecan level, i.e., the sum of intact aggrecan and degraded aggrecan, in the joint fluid. It has been well established by other researchers that the release of aggrecan from degenerated cartilage was inhibited by Na-HA^{33,35}; therefore, the results shown in Fig. 2 indicate the inhibitory effect of Na-HA on the release of aggrecan from the cartilage of patients with knee OA.

On the other hand, even though the level of CS reflecting total aggrecan was significantly decreased, the level of intact aggrecan, that was aggregating with HA, was stable during the series of Na-HA injections (Fig. 3). This means the level of degraded aggrecan alone was reduced in the joint fluid by the series of Na-HA injections. We interpret these results shown in Figs. 2 and 3 indicate an inhibitory effect of Na-HA on the release of aggrecan from the cartilage and also an up-regulation by Na-HA of the intact aggrecan synthesis and/or a down-regulation by Na-HA of intact aggrecan degradation in the cartilage and in the joint fluid. Both of these regulations increase the amount of intact aggrecan in the cartilage, and

must lead to an increase of intact aggrecan level in the joint fluid when the release rate of intact aggrecan is stable. However, because the release rate of intact aggrecan was reduced by Na-HA injection, the level of intact aggrecan did not change significantly in the joint fluid. In other words, our interpretation is that the decrease in level of released intact aggrecan, which is induced by the inhibitory effect of Na-HA on the release of aggrecan, is canceled by the increase in level of released intact aggrecan, which is induced by the up-regulation of the intact aggrecan synthesis and/or the down-regulation of intact aggrecan degradation.

Some researchers have reported that Na-HA accelerated aggrecan synthesis after it penetrated into degenerated cartilage derived from human^{27,29}, bovine¹⁸, and horse²⁸. This finding supports the possibility of the up-regulation by Na-HA of the intact aggrecan synthesis in the cartilage of patients treated in this study. In the other papers, Na-HA is reported to (1) have a suppressive effect on the gene expression of MMP-3 in the synovium during development of OA in a rabbit model³⁶, and to (2) stimulate the production of TIMP-1 from bovine articular chondrocytes, thus, reducing the stromelysin/TIMP-1 ratio²⁴. These results suggest the down-regulation by Na-HA of the intact aggrecan degradation in the cartilage and also in the joint fluid. Therefore, we consider that the results shown in Figs. 2 and 3 reflect the complex effects of Na-HA, i.e., the inhibitory effect on aggrecan release from the cartilage, the up-regulation of intact aggrecan synthesis in the cartilage, and/or the down-regulation of intact aggrecan degradation in the cartilage and in the joint fluid of patients with knee OA.

It is well known that NO has many physiological functions. Several reports also implicate NO in the pathophysiology of OA, including the elevation of nitrite levels in the joint fluids of patients with OA and rheumatoid arthritis³⁷ and in both the serum and joint fluid of patients with OA^{37,38}. A recent study has shown elevated NO levels in the joint fluid from patients with temporomandibular disorders caused by OA³⁹. In addition, unlike chondrocytes recovered from normal human articular cartilage, chondrocytes recovered from patients with OA produce NO spontaneously⁴⁰ and express NO synthase⁴¹. Based on these findings, the possibility exists that NO is involved in the proteolytic activity in the joint tissues, upstream of the pathological process and the symptoms of OA, such as joint inflammation. Because recent studies on experimental OA induced by anterior cruciate ligament transection or partial meniscectomy in rabbits showed that Na-HA significantly reduced NO synthesis from the menisci and synovium^{30,31}, we evaluated the effect of Na-HA on NO levels in the joint fluids of OA patients. The present study demonstrates that the intra-articular injection of Na-HA significantly decreased the NO level in the joint fluid of the OA knee (Fig. 4). Therefore, we conclude that Na-HA exerted its effect on joint fluid NO levels as a result of the down-regulation of NO production by the menisci and/or synovium in the patient with OA.

The authors hypothesize that NO joint fluid levels will be linked with the degradation of aggrecan and also with the symptoms of OA patients, as described above. Therefore, we looked for any correlations among the joint fluid levels of NO, intact aggrecan and CS, and also with the clinical score. However, contrary to our expectations, there were no significant correlations between joint fluid NO levels and the other parameters analyzed (Table 1). On the other hand, there were tendencies ($P < 0.1$) for positive correlations between the clinical score and C4S or C6S levels. Thus, the improvement of clinical symptoms by Na-HA is partially based on the prevention of proteoglycan release from the

Table I
Correlation coefficients among study parameters: the clinical score and joint fluid levels of C4S, C6S, intact aggrecan and NO

	C4S	C6S	Intact aggrecan	NO
Clinical score	0.446 ($P < 0.1$)	0.433 ($P < 0.1$)	0.248 (NS)	0.239 (NS)
C4S			−0.426 ($P < 0.1$)	
C6S	0.892 ($P < 0.001$)		−0.427 ($P < 0.1$)	
NO	0.052 (NS)	0.031 (NS)	0.076 (NS)	

Number of patient: 15 or 16, NS: not significant.

cartilage, whereas the level of NO in joint fluid may be reduced independently from the effects of Na-HA on the prevention of aggrecan release or clinical symptoms.

In summary, the present study showed the possibility of a suppressive effect on the release and degradation of aggrecan and/or an accelerative effect on the synthesis of aggrecan, as well as a suppressive effect on NO production in the joint tissues of OA patients treated with a series of Na-HA injections. This may be an important mechanism for the activity of Na-HA during the development of OA. The significance and actual relationship of these two effects will be important subjects in future studies.

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